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Suppressive Effects of Transforming Factor-β and Interleukin-10 on the Cytolytic Activity of Murine Macrophages and Reversal by Cytokines

Chin-Hung Lin

ABSTRACT

Suppressive Effects of Transforming Growth Factor-β and Interleukin-10 on the Cytolytic Activity of Murine Macrophages and Reversal by Cytokines

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In this study, the suppressive effects of transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) on peritoneal macrophage killing of H238 target cells and the potential for reversal of the immunosuppressive effect by IL-4 and interferon-γ (IFN-γ) were investigated. The responsiveness of naive and peptone-activated macrophages was compared. The cytolytic activity for tumor cells of these effector cells was measured by percent lysis of ^{3}H-thymidine labeled Herpes simplex virus type 2-transformed tumor cells (H238). After 18-24 hours of incubation with TGF-β or IL-10, the cytolytic activity of macro-

medium alone. The immunosuppressive effect of TGF-β or IL-10 on non-activated macrophages was dose-dependent, with as little as 1 ng/ml TGF-β or 25.0 ng/ml IL-10, suppressing the cytolytic activity. After coincubation with TGF-β (5ng/ml) and IL-4 (200ng/ml) or IFN-γ (400 unit/ml), inhibition in the killing activity of macrophages was reversed by 20-30% when compared to controls incubated with TGF-β alone. IL-4 and IFN-γ both partially reversed the immunosuppressive effects of TGF-β on macrophages. When 10% proteose peptone was injected into mice to activate macrophages, the findings show that TGF-β could suppress non-activated macrophage cytotoxicity, but did not inhibit the cytotoxicity of peptone-activated cells. The killing activity of resting macrophages was significantly inhibited by 50 ng/ml of IL-10. When H238 tumor-bearing mice were treated with anti-TGF-β antibody (Ab), the results show that the production of tumor necrosis factor-α (TNF-α), a cytokine known to directly destroy tumors, in H238 tumor-bearing mice without treatment is lower than in H238 tumor-bearing mice with anti-TGF-β Ab treatment.

LOMA LINDA UNIVERSITY

Graduate School

SUPPRESSIVE EFFECTS OF TRANSFORMING FACTOR-β AND INTERLEUKIN-10 ON THE CYTOLYTIC ACTIVITY OF MURINE MACROPHAGES AND REVERSAL BY CYTOKINES

by

Chin-Hung Lin

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Each person whose signature appears below certifies that this thesis in his/her opinion is adequate, in scope and quality, as a thesis for the degree Master of Science.

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INTRODUCTION

Transforming growth factors (TGF) are a family of peptides that can reversibly induce non-neoplastic cells to express the transformed phenotype as characterized by the acquisition of anchorage-independent growth and by the loss of density-dependent inhibition of growth. Two types of TGF, type-α TGF and type-β TGF, have been identified. In the presence of both TGF-α and TGF-β, normal rat kidney fibroblasts can be stimulated to grow under anchorage independent conditions as demonstrated by their ability to grow in soft agar (Frolik et al. 1984). In addition, a variety of virus-transformed and certain chemically-transformed cells are able to produce TGF-α, which is very similar to epidermal growth factor, and TGF-β in order to maintain the transformed phenotype (Roberts and Frolik, 1983). Since these phenomena suggest a potential relationship between the biological activity of TGF and the events that occur during malignant transformation, it is important to investigate the effects of TGF on those cells of immune system which

function against malignant transformation. Some studies have shown that TGF-α has little effect on lymphocytes. TGF-β is now known to be a powerful immunomodulatory agent.

TGF-β consists of a family of Mr 25-kDa homodimeric proteins secreted by a variety of transformed and nontransformed cells, including platelets, lymphocytes, and macrophages. It is also produced by many other tumor cell types including murine MH134 hepatoma, A-549 and PC-9 human lung adenocarcinoma, human colon tumor, etc. Once activated, this cytokine binds to a unique receptor found on virtually all cells. Several forms of TGF-β have been identified. Some studies have shown that TGF-β1 and TGF-β2 have differential activity in endothelial cells; and TGF-β2 is much less potent than TGF-β1 in inhibiting DNA synthesis in aortic endothelial cells (Jennings et al. 1988). Recently, TGF-β has been demonstrated to exert profound inhibitory effects on IL-2-dependent T cell proliferation; IL-1-dependent murine thymocyte proliferation; and B cell proliferation and immunoglobulin secretion (Kehrl et al. 1986).

In addition to TGF-β, interleukin-10 also play a major role in down-regulation of immunologic reactions. IL-10 is 18.5 kDa polypeptides and was originally named cytokine synthesis inhibitory factor (CSIF) due to its ability to inhibit synthesis of IFN-γ and other cytokines in peripheral blood mononuclear cell cultures. It is produced by the Th2 subset of $CD4^+$ T lymphocytes (Fiorentino and Bond, 1989), Ly-1^+ B cells (O'Garra et al. 1990), and mast cells and has been shown to inhibit Th1 cells secretion of certain cytokines including IL-2 and IFN-γ which preferentially induce macrophage activation, and acts as a growth stimulatory factor together with IL-2 and IL-4 on mature and immature T cells (Cher et al. 1987). Some studies have also shown that IL-10 reduces H_2O_2 and NO production by macrophages (Gazzinelli et al. 1992). Hence, both TGF-β and IL-10 may exert immunosuppressive effects through regulation of cytokine production.

Interferon-γ is produced by Th1 cells. Some studies have reported that IFN-γ induces class I and class II MHC antigen on

many cells, and enhances IgG_{2a} production. Schreiber et al. (1985) also showed that induction of the tumoricidal response required the interaction of IFN-γ with a specific cell surface receptor on macrophages. Interleukin-4 is produced by Th2 cells. It has been shown to promote T cell growth IgG and IgE secretion by B cells, and production of mast cell growth factor. Crawford et al. (1987) reported that IL-4 promotes the differentiation of macrophages into a cytotoxic phenotype for increased tumoricidal activity. Such studies indicate that IL-4 and IFN-γ are important regulators of macrophage function.

Generally, cancer is associated with immunosuppression, but it might not necessarily render the host immune system totally incompetent. The study of factors that affect the immune response of individuals is very complex. Some studies have shown the effects of TGF-β on lymphocyte activity, neutrophil, and natural killer (NK) cell function. Our laboratories have been studying the immunosuppression derived from human

B-lymphoblastoid and melanoma cell lines (Repique et al. 1992), and the immune response of BALB/c mice to herpes simplex virus Type-2 transformed syngeneic tumor (H238) (Prabhu Das et al. 1991). This last study by Prabhu Das et al. (1991) has shown that TGF-β appears to be a main inhibitor of immune responses found in the H238 tumor cell line. IL-10 also appears to inhibit cytokine responses. In order to understand better the effect of TGF-β, IL-10 and other cytokines, one purpose of this study was to test the inhibitory effects of TGF-β and IL-10 on murine macrophages. Another purpose of this study was to investigate if cytokines with known immunoenhancing abilities could prevent and/or reverse any inhibition.

In this study, we show that TGF-β and IL-10 inhibit H238 tumor cell killing activity of resting intraperitoneal macrophages. Once macrophages are activated by proteose peptone in vivo, the cytolytic activity of this cytotoxic phenotype macrophage is not suppressed by TGF-β. IL-4 and IFN-γ both partially reversed the immunosuppressive effects of TGF-β on non-acti-

vated macrophages.

MATERIALS AND METHODS

Animals

Male BALB/cByJ mice were obtained from the breeding colony at our animal facility at 10-14 weeks of age. The animals were housed in stainless steel cages with 5 mice/cage in a room controlled for temperature, humidity and a 12-hour light/dark cycle, Some mice were purchased from Jackson Laboratories, Inc. (Bar Harbor, MA).

Effector Cells: Macrophages

The resting macrophages from untreated, non-tumor-bearing BALB/c mice were collected after intraperitoneal (i.p.) injection of 8 to 10 ml of Dulbecco's minimum essential medium (DMEM; Irvine Scientific, Santa Ana, CA, USA) that contained 10% fetal calf serum (FCS, HyClone Laboratories, Inc. Logan, UT), 50 ug/ml gentamicin (Sigma Chemical Co, St. Louis, MO, USA), and 100 ug/ml fungizone (E.R. Squibb and Sons, Inc., Princeton, NJ, USA). Peritoneal fluid was withdrawn through

the abdominal wall with a 20 gauge needle and 10 ml syringe and was pooled in 60 x 15mm tissue culture dishes. After 2 hours incubation at 37°C in 5% CO_2, nonadherent peritoneal cells (PC) were removed from the adherent population by repeated gentle washing with DMEM. Macrophages were detached from the dishes by gently using a cell scraper and washed with complete DMEM. After washing, these cells were adjusted to 1 x 10^6 macrophages/ml in complete DMEM. For the inflammatory or activated macrophages, the cells were obtained from mice that had been injected i.p. 48 hours earlier with 10% proteose peptone (DIFCO, Detroit, MI) in a volume of 1 ml/mouse. These cells were also collected, washed, and adjusted to 1 x 10^6 macrophages/ml as described above.

Target Cells: H238 Tumor Cells

The H238 cells were obtained from Dr. A.L. Boyd (Frederick, MD, USA). They were originally developed by Boyd and Orme by exposing BALB/c mouse embryo fibroblasts to UV-irradiated HSV-2 (Savage strain). Prior to testing, the cells were

passaged several times in DMEM supplemented with 10% defined bovine calf serum (BCS; Hyclone Laboratories, Logan, UT, USA), 100 ug/ml fungizone, and 50 ug/ml gentamicin at 37° C in 5% CO_2.

Tumor Induction and Volume

The H238 tumor cells were harvested during exponential growth (for phase I of study) or at the time of confluent monolayer (for phase II of study) and counted using the trypan blue exclusion method immediately prior to subcutaneous injection of 1 x 10^6 cells into the right thigh. Although this dose of H238 cells consistently produces progressively growing fibrosarcomas in >95% of mice, exponentially growing cells will produce more rapidly growing tumor than monolayered cells. Tumor development was monitored by taking 3-dimensional measurements with vernier calipers 2-3 times each week. The tumor size was calculated using a formula which approximates the volume of a hemiellipsoid as follows:

$$\text{Tumor volume}(mm^3) = L \times W \times H/2$$

where L is length, W is the width, and H is the height of the tumor.

Cytokines

Human recombinant TGF-β1 and recombinant murine IL-4 were purchased from R & D Systems (Minneapolis, MN, USA). The purity of TGF-β1 was >95% and it had an ED_{50} of 110 pg/ml. IL-4 had >97% purity and an ED_{50} of 1-2 ng/ml. Murine recombinant forms of IFN-γ and IL-10 were obtained from Genzyme (Cambridge, MA). The IFN-γ had >95% purity and specific activity of 1×10^7 units/mg. The purity of IL-10 was >98%; its specific activity was 5×10^5 units/mg.

Anti-TGF-β Antibody

Rabbit polyclonal anti-TGF-β was purchased from R & D Systems, Inc. in lyophilized form. Approximately 10 ug of this antibody will neutralize 250 pg of TGF-β1 and an equivalent amount of the TGF-β2 and β3 isoforms (personal communication, Steve Weiland, Technical consultant, R & D Systems).

The antibody was reconstituted with sterile saline, aliquoted, and stored in a conventional freezer until just prior to use.

The Effect of TGF-β and Anti-TGF-β Antibody on H238 Tumor Volume Assay

In the first phase 11 mice were injected with the H238 cells (harvested during exponential growth) and all animal were randomized into 3 groups with 3-7 mice/group: a) no tumor and no treatment control, b) tumor and no treatment, and c) tumor + anti-TGF-β antibody. Treatments were begun 1 day after injection of the tumor cells. The anti-TGF-β antibody was given 9 times over a 10-day period at 100 ng/mouse/day for a total of 900 ng/animal. The mice were euthanized for assay 18 days after H238 cell injection (8 days after the last treatment). In phase II of the study, 15 mice were injected with H238 cells (harvested at the time of confluent monolayer) and randomized into the same groups (5-10 mice/group) as in phase I. Treatments were again initiated 1 day after injection of the tumor cells. The anti-TGF-β antibody was given once per day at 100 ng/mouse/injection for a total of 9 injections over the same

10-day period. The mice were euthanized at 16 days after H238 cell injection (6 days after the end of anti-TGF-β treatment). In both phases, all treatments were injected i.p.

Macrophage-Induced H238 Tumor Cytotoxicity Assay

For the cytotoxicity assay, H238 cells were obtained from trypsin-digested monolayers. These cells (2 to 5 x 10^6 viable cells/20 ml culture media) were incubated with 0.25 uCi/ml ^{3}H-TdR (sp. act. 5.0 Ci/mmol, ICN Radiochemicals, Irvine, CA) in 75-cm^2 culture flasks for 18-24 hours. Macrophages (1 x 10^6 cells/ml) were incubated with TGF-β (1.0 ng/ml, 2.5 ng/ml, 5.0 ng/ml, 10 ng/ml and 20 ng/ml) alone or without TGF-β as control, and with IL-10 (0.5 ng/ml, 5.0 ng/ml, 10 ng/ml, 25 ng/ml, and 50 ng/ml) alone or without IL-10 as control for 18-24 hrs. After incubation, H238 cells labeled with ^{3}H-TdR were obtained from trypsin-digested monolayers and washed with DMEM for three times to remove unincorporated label. ^{3}H-labeled H238 cells were then adjusted to 2 x 10^5 cells/ml. One hundred ul of pretreated macrophages and 100 ul of labeled

H238 cells were added to 96-well flat-bottomed microtiter plates [2 x 10^4 target (T) cells/well, 1 x 10^5 effector (E) cells/well]. Effector:target (E:T) cell ratio was 5:1. One hundred ul of labeled H238 cells were incubated with either 100 ul of DMEM as spontaneous release control or 100 ul of SDS as total release control. After 18-24 hours incubation, 100 ul of supernatant was collected from each well and added into each 6 ml mini poly-Q vial tube (Beckman, Irvine, CA); then 3 ml of cytoscint (ICN Biocmedicals Inc.) was added into each vial tube. The cytolytic activity of macrophages was determined by measurement of the ^{3}H-TdR released into culture supernatant, and was expressed as percent as follows:

$$\% \text{ Killing} = \frac{\text{Experimental Release - Spontaneous Release}}{\text{Total Release - Spontaneous Release}} \times 100$$

The Reversal Effect By Cytokines Assay

Macrophages (1 x 10^6 cells/ml) were incubated with IFN-γ (0.4ng/ml, 4.0 ng/ml, 40 ng/ml and 400 ng/ml) or IL-4 (0.2 ng/ml, 2.0 ng/ml, 20 ng/ml and 200 ng/ml) in the presence or

absence of 5.0 ng/ml TGF-β. When two cytokines were used, they were both added simultaneously to the cells. One hundred ul of labeled H238 cells (2 x 10^5/ml) and 100 ul of pretreated macrophages were added to the 96-well flat-bottomed microtiter trays. One hundred ul of labeled H238 cells were incubated with either 100 ul of DMEM as spontaneous release control or 100 ul of SDS as total release control. After 18-24 hours incubation, the cytolytic activity of macrophage was measured by the ^{3}H-TdR released into culture supernatant and was expressed as a percentage. Supernatant were collected and counted as described above. The formula was the same as that given above.

The Effect of Anti-TGF-β Antibody on TNF-α Production Assay

BALB/c mice were divided into 3 groups. Each group contained 5-10 mice each. Group A consisted of untreated normal mice (negative control); group B was tumor-bearing mice without any treatment (positive control); group C was tumor-bearing mice with anti-TGF-β antibody treatment. Blood was collected from each mouse by cardiac puncture and plasma levels of

TNF-α were determined using a standard enzyme-linked immunosorbent assay. Alquots (50 ul) of each test sample and TNF-α standards (R & D Systems, Inc.) were prepared and dispensed into pre-coated with anti-TNF-α Ab 96-well stripwell microtiter plates and incubated for 2 hours at 37°C and washed 5 times with washing buffer. Pre-diluted conjugate reagent was added at 100 ul/well, the plates were reincubated for 1 hour, and washed again 5 times. OPD substrate reagent was dispensed at 100 ul/well and the color reaction was allowed to develop in the dark at room temperature for 30 min. The reaction was stopped by addition of 100 ul of 2M sulfuric acid and the absorbance was determined in an EIA plate reader at 490 nm. All reagents for this assay were obtained from Endogen, Inc. (Boston, MA).

Statistical Analysis

Results were analyzed by one-way analysis of variance (ANOVA) and Tukey's honestly significant difference multiple range test at $p < 0.05$ using STATGRAPHICS 3.0 (STSC, Inc.,

Rockville, MD).

RESULT

The Effects of TGF-β and Anti-TGF-β Antibody on H238 Tumor Volume

The mean tumor volumes of mice in phase I and phase II are presented in Figures 1 and 2, respectively. All mice, regardless of treatment, developed progressively growing tumors. As expected, more rapidly growing tumor were induced in the animals injected with exponentially growing cells compared to those injected with cells which were harvested while in confluent monolayer. The mice with anti-TGF-β antibody treatment developed growing tumor as rapidly as the mice without anti-TGF-β antibody treatment in both phase I and II.

The Effect of TGF-β on Murine Resting Macrophages

The cytolytic activity of resting peritoneal macrophages from untreated mice was tested by the destruction of H238 tumor cells in the presence or absence of TGF-β. The mean dpm ± S.E. (standard error of the mean) and % killing activity are shown Figure 3 and Table 2 (Appendix). These data show that

the immunosuppression of TGF-β on inactivated macrophages is dose-dependent, with as little as 1 ng/ml TGF-β, significantly inhibiting the cytolytic activity of macrophages ($p < 0.05$).

The Effect of TGF-β on Peptone-Activated Murine Macrophages

Peptone-activated macrophages were obtained from non-tumor-bearing mice that were injected 2 days earlier with 10% proteose peptone. The mean dpm ± S.E. and % killing activity are shown in Figures 4A and 4B and Table 3 (Appendix). The results suggest that once macrophages are activated by proteose peptone in vivo, the cytolytic activity of these active macrophages would not be inhibited by TGF-β even when the concentration of TGF-β is up to 20 ng/ml. The killing activity of primed macrophages is approximately 15% greater than that of resting macrophages in the same age mice.

The Effect of IL-10 on Murine Resting Macrophages

Resting macrophages were incubated with 0.5 ng/ml, 5.0 ng/ml, 10 ng/ml, 25 ng/ml, and 50 ng/ml IL-10 for 18-24 hours.

The result is shown in Figure 5 and Table 4 (Appendix). Our finding shows that the cytolytic activity of macrophages is significantly inhibited by 50 ng/ml of IL-10. The suppressive effect of IL-10 on resting macrophages is also dose-dependent manner.

The Reversal of Inhibitory Effect of TGF-β on Macrophages by IFN-γ or IL-4

Resting macrophages were treated with 5 ng/ml TGF-β and different concentrations of IFN-γ or IL-4 were added simultaneously. Figure 6 and Table 5 (Appendix) show the mean dpm ± and % killing activity. The results suggest that IFN-γ and IL-4 both partially reversed the immunosuppressive effects of TGF-β on macrophages, but statistical significance was not obtained.

The Effect of TGF-β on TNF-α Production

Tumor-bearing mice were administered i.p. with a total of 900 ng anti-TGF-β 1-10 days after injection with 1×10^6 H238 tumor cells/mouse. Table 1 shows the mean plasma levels of TNF-α from each group of mice. The results show that tumor-bearing mice produce significantly more TNF-α than normal

controls without tumor. Although tumor-bearers treated with anti-TGF-β Ab had slightly higher plasma TNF-α levels than their nontreated counterparts, statistical support was lacking.

Figure 1. Tumor volumes for mice in phase I after injection of 1 x 10^6 H238 tumor cells harvested in exponential growth. The mean ± S.E.M. are shown for 3-7 mice/group. B = no treatment; C = anti-TGF-β. The result shows that i.p. administration of polyclonal anti-TGF-β Ab did not inhibit H238 tumor progression in vivo.

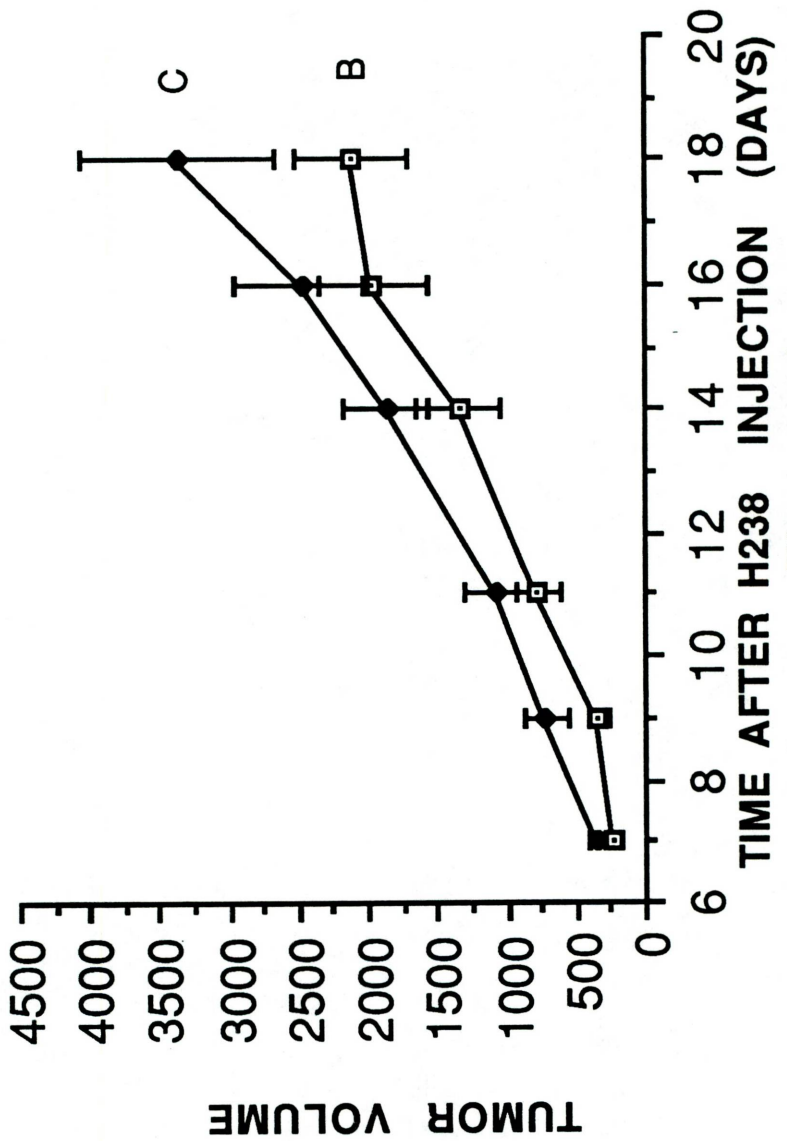
TUMOR VOLUME
4500
4000
3500
3000
2500
2000
1500
1000
500
0
6
8
10
12
14
16
18
20
TIME AFTER H238 INJECTION (DAYS)
C
B

Figure 1

Figure 2. Tumor volumes for mice in phase II after injection of 1 x 10^6 H238 tumor cells harvested while in monolayer. The mean ± S.E.M. are shown for 5-10 mice/group. B = no treatment; C = anti-TGF-β.

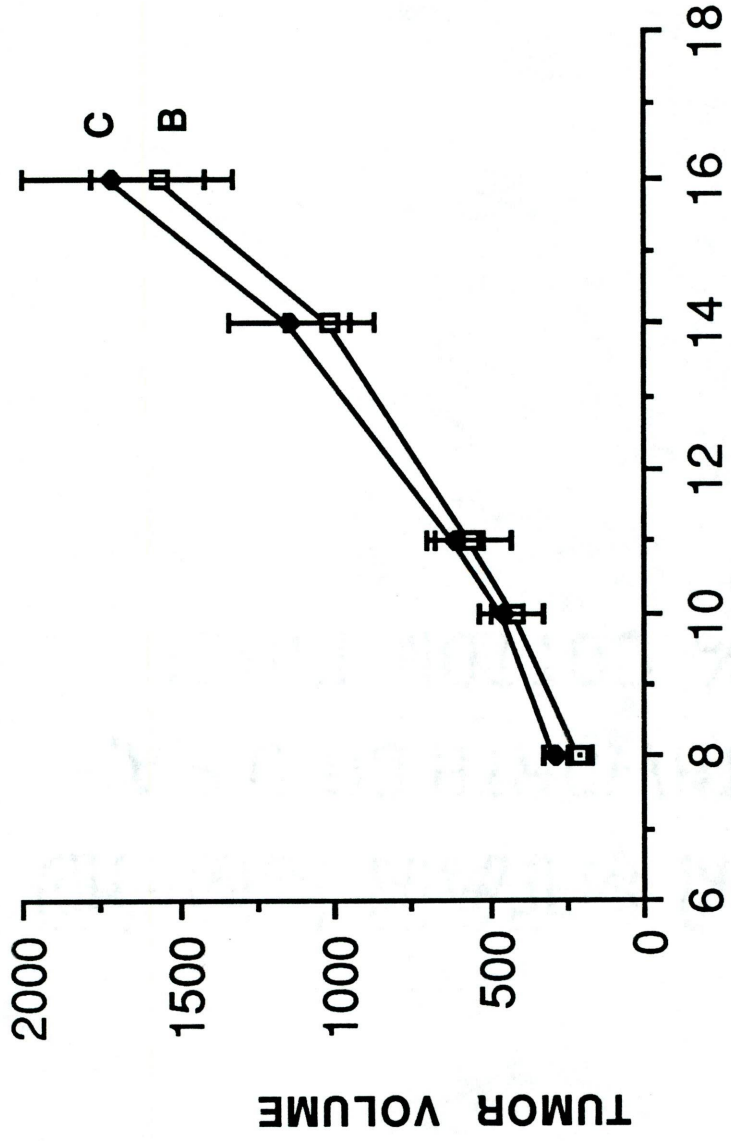
TUMOR VOLUME
2000
1500
1000
500
0
6
8
10
12
14
16
18
TIME AFTER H238 INJECTION (DAYS)
C
B

Figure 2

Figure 3. The suppressive effect of TGF-β on murine resting macrophages. The cells were obtained from 11 week old BALB/c mice and were incubated with 1 ng/ml, 2.5 ng/ml, 5.0 ng/ml, 10.0 ng/ml and 20.0 ng/ml TGF-β, and with medium alone as control for 18-24 hrs before testing their cytolytic activity. The killing activity was measured by % lysis of ^{3}H-TdR labeled H238 cells. The killing activity of resting macrophages was significantly decreased ($p < 0.05$) in the presence of TGF-β as indicated by the asterisks.

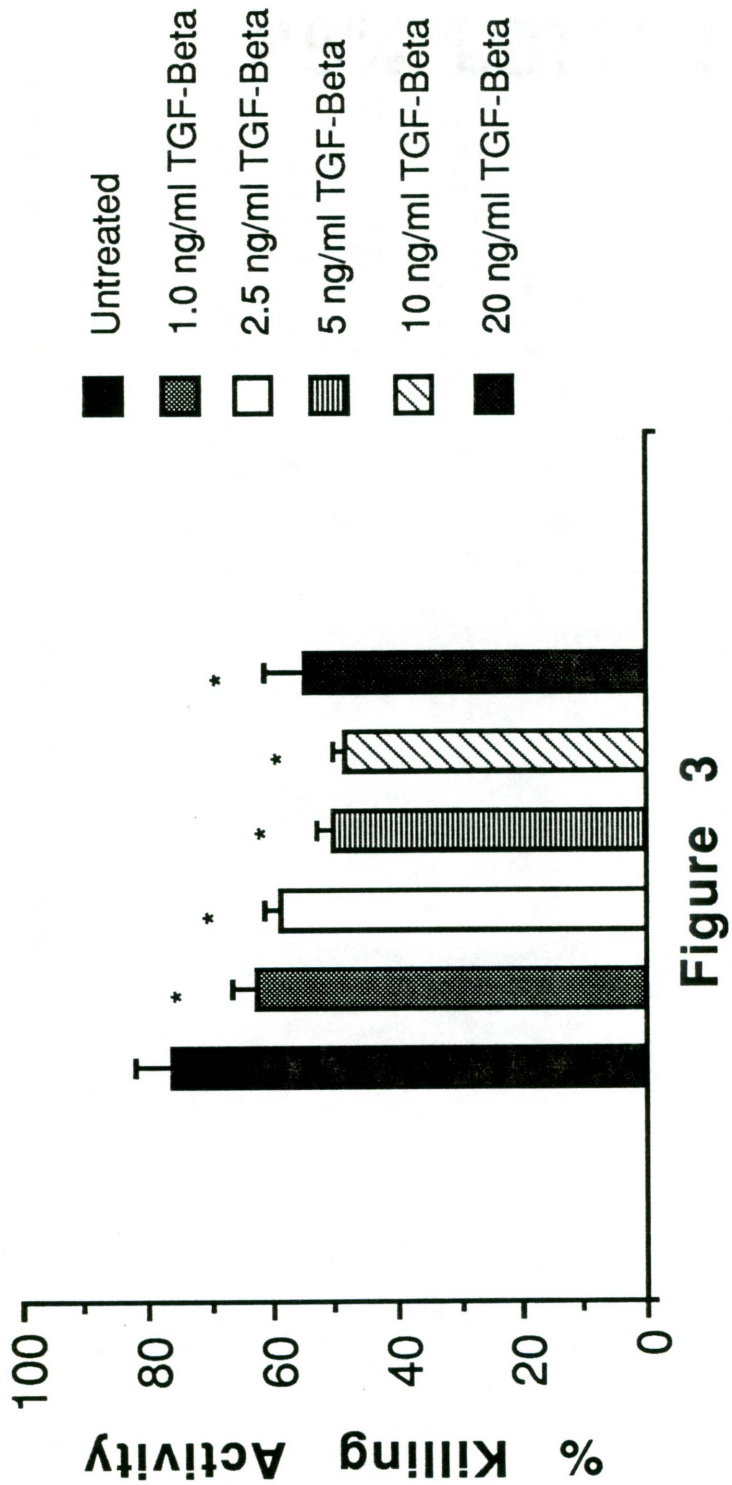
Untreated
1.0 ng/ml TGF-Beta
2.5 ng/ml TGF-Beta
5 ng/ml TGF-Beta
10 ng/ml TGF-Beta
20 ng/ml TGF-Beta
% Killing Activity
100
80
60
40
20
0
*
*
*
*
*
*

Figure 3

Figure 4. The effect of TGF-β on proteose peptone-activated macrophages (Fig. 4A). The inflammatory macrophages were obtained from 9 week-old BALB/c mice injected 2 days earlier with 10% proteose peptone. These active macrophages were incubated with 1.0 ng/ml, 2.5 ng/ml, 5.0 ng/ml, 10 ng/ml, 25.0 ng/ml TGF-β, and medium alone as control for 18-24 hrs. The killing activity was measured by % lysis of ^{3}H-TdR labeled H238 cells. The result shows that TGF-β had little or no suppressive effect on these proteose peptone-activated macrophages. The effect of TGF-β on resting macrophages (Fig. 4B). The resting macrophages were obtained from untreated 9 week-old BALB/c mice. The macrophage treatment is the same as that for proteose peptone-activated cells in Fig. 4. The result shows that the killing activity was significantly inhibited by TGF-β. The cytolytic activity of active macrophages is better than that of resting macrophages in the same age mice. *Significantly different from untreated macrophages ($p < 0.05$).

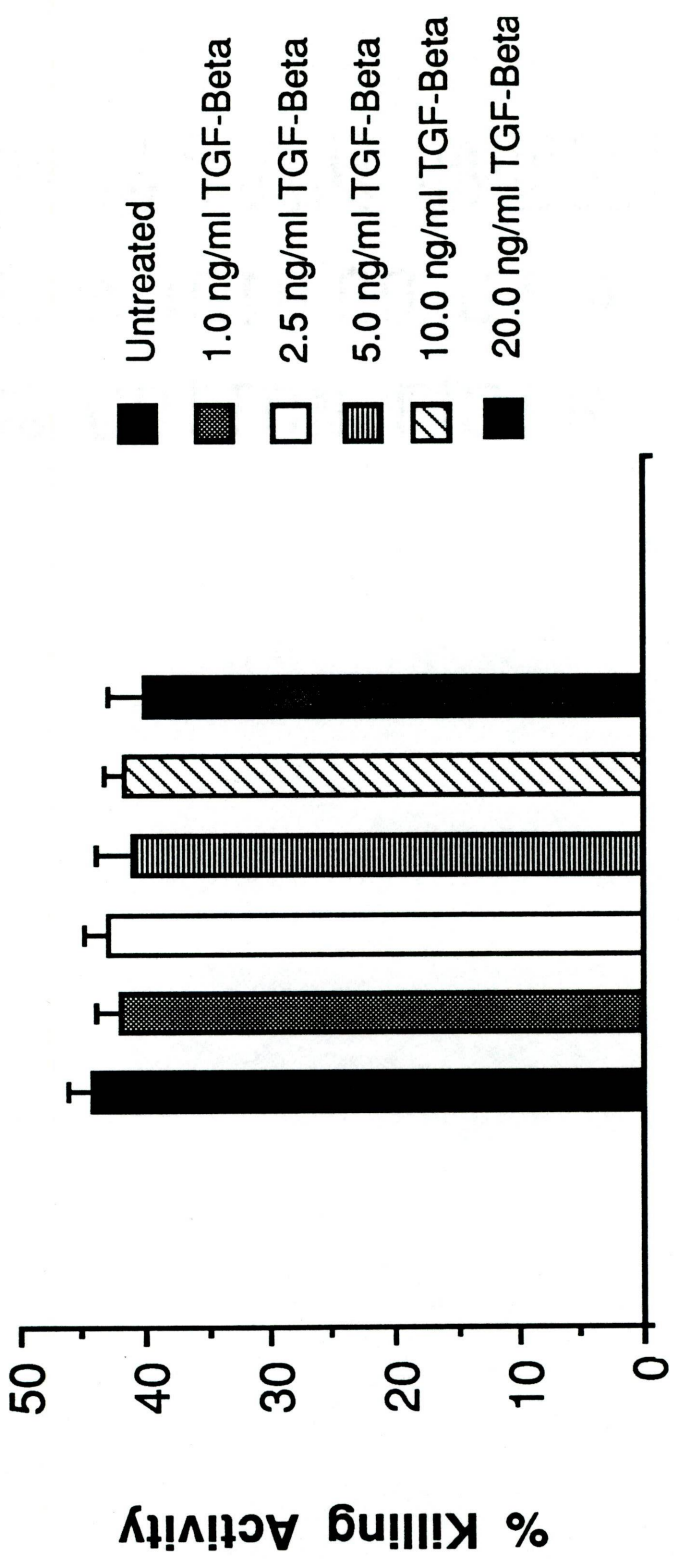
Untreated
1.0 ng/ml TGF-Beta
2.5 ng/ml TGF-Beta
5.0 ng/ml TGF-Beta
10.0 ng/ml TGF-Beta
20.0 ng/ml TGF-Beta
% Killing Activity
0
10
20
30
40
50

Figure 4A

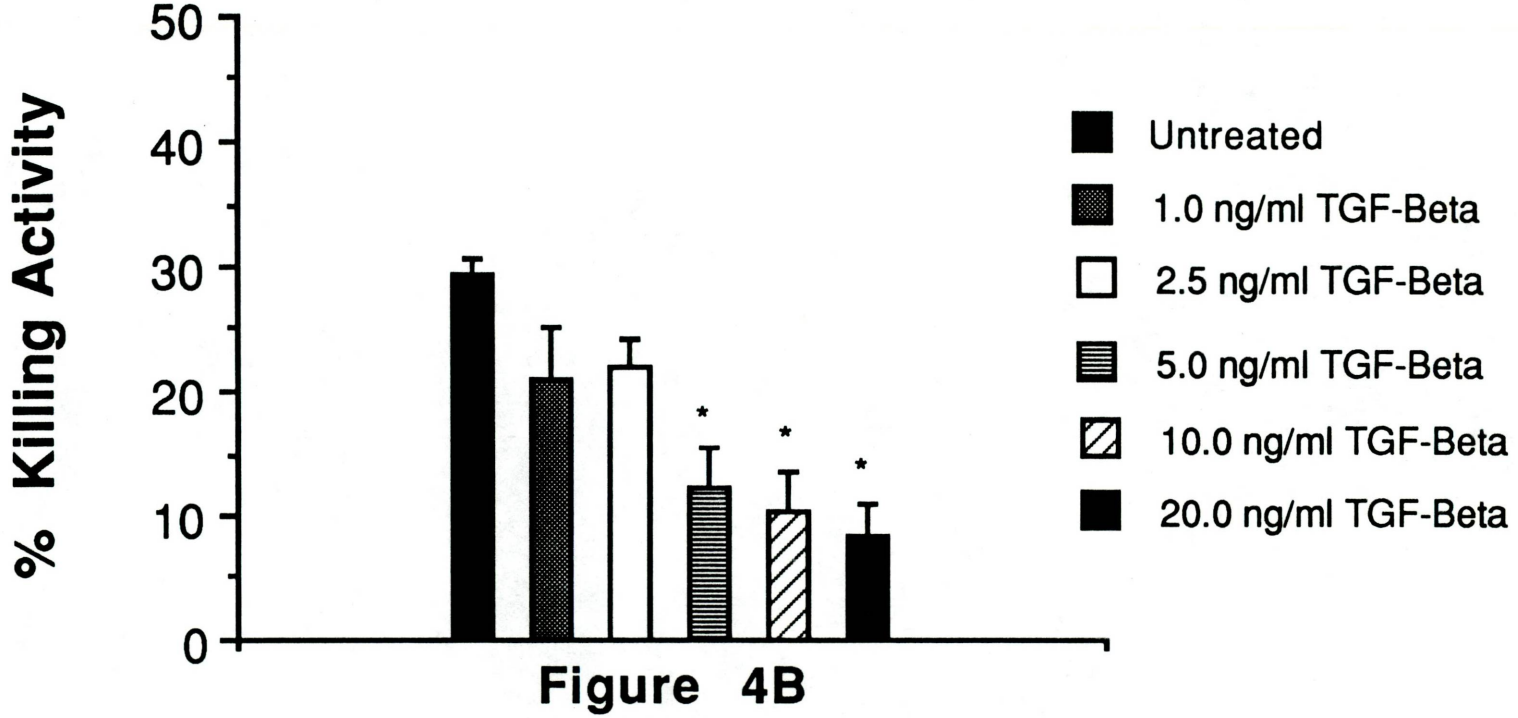
% Killing Activity
50
40
30
20
10
0
*
*
*
Untreated
1.0 ng/ml TGF-Beta
2.5 ng/ml TGF-Beta
5.0 ng/ml TGF-Beta
10.0 ng/ml TGF-Beta
20.0 ng/ml TGF-Beta

Figure 4B

Figure 5. The effect of IL-10 on resting macrophages. Resting macrophages were obtained from untreated 12 week-old BALB/c mice. The resting macrophages were incubated with 0.5 ng/ml, 5.0 ng/ml, 10.0 ng/ml, 25.0 ng/ml, and 50 ng/ml TGF-β respectively for 18-24 hrs. The results show that the cytolytic activity of resting macrophages was significantly decreased at the concentration of 50 ng/ml IL-10 as indicated by the asterisk ($p < 0.05$).

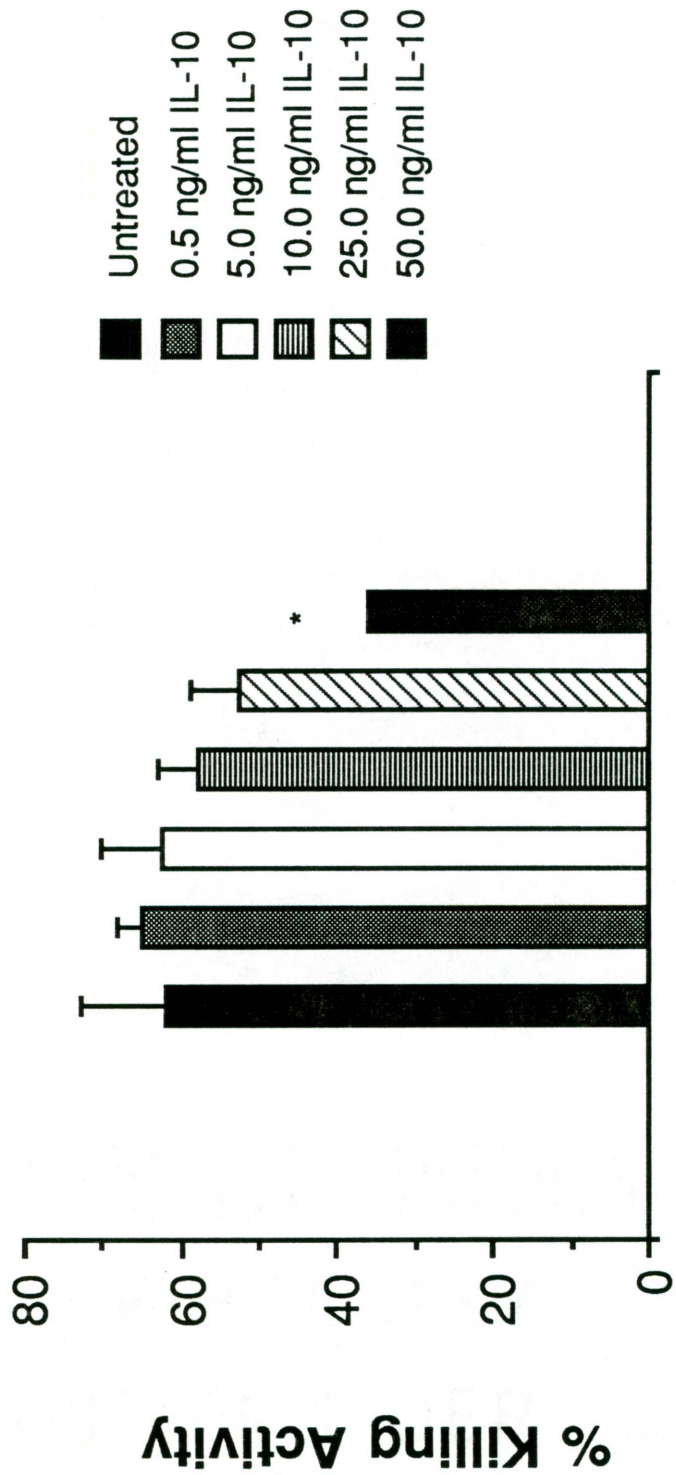
Untreated
0.5 ng/ml IL-10
5.0 ng/ml IL-10
10.0 ng/ml IL-10
25.0 ng/ml IL-10
50.0 ng/ml IL-10
*
% Killing Activity
0
20
40
60
80

Figure 5

Figure 6. The reversal of inhibitory effect of TGF-β on resting macrophages by IFN-γ or IL-4. The resting macrophages were incubated with 5 ng/ml TGF-β and IFN-γ (400 ng/ml, 40 ng/ml, 4 ng/ml, and 0.4 ng/ml respectively) or IL-4 (200 ng/ml, 20 ng/ml, 2 ng/ml, and 0.2 ng/ml) simultaneously for 18-24 hrs. The killing activity of resting macrophages was measured by % lysis of ^{3}H-TdR labeled H238 cells. The results suggest that IL-4 and IFN-γ both could partially reverse the inhibitory effect of TGF-β on macrophages, but statistical support was weak; and the reversal effects of IL-4 and IFN-γ are dose-dependent. *Significantly different compared to untreated control ($p < 0.05$).

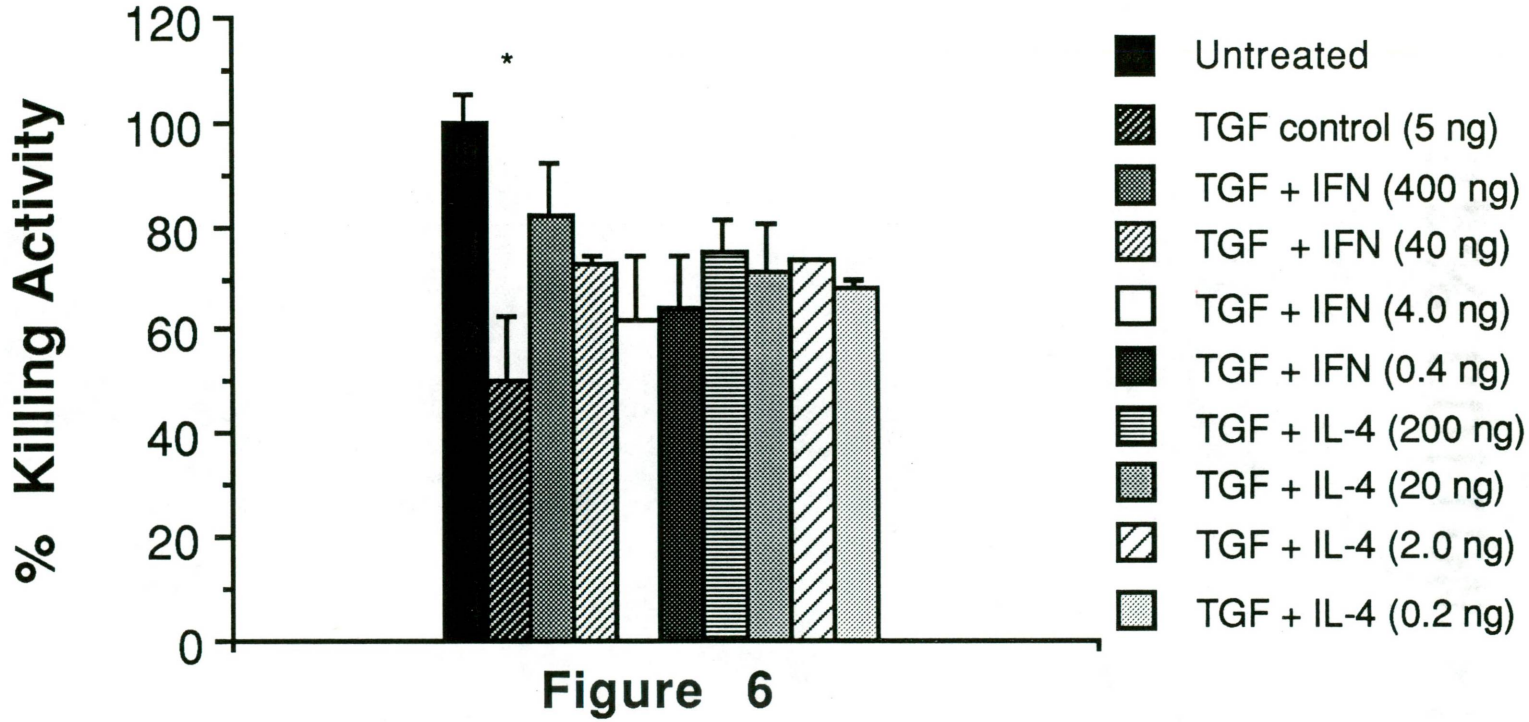
% Killing Activity
120
100
80
60
40
20
0
*
Untreated
TGF control (5 ng)
TGF + IFN (400 ng)
TGF + IFN (40 ng)
TGF + IFN (4.0 ng)
TGF + IFN (0.4 ng)
TGF + IL-4 (200 ng)
TGF + IL-4 (20 ng)
TGF + IL-4 (2.0 ng)
TGF + IL-4 (0.2 ng)

Figure 6

Table 1. The Effects of TGF-β on TNF-α Production

Mice Treatment	Plasma levels of TNF-α	
	Phase I[a]	Phase II[a]
No tumor + no treatment	0.8 ± 0.3[b]	0.9 ± 0.5
Tumor + no treatment	39.2 ± 8.3[c]	14.1 ± 4.6
Tumor + anti-TGF-β Ab	49.7 ± 5.6[c]	19.7 ± 8.8

[a]Phase I and phase II = 900 ng/ml of anti-TGF-β(100 ng/ml) were administrated i.p. over a 10-day period beginning 1 day after H238 tumor cell injection. The mice were euthanized 18 days after tumor injection.

[b]Mean ± S.E.; units/ml.

[c]Significantly higher than no tumor control group ($p < 0.05$).

DISCUSSION

The immunosuppression associated with malignant disease has been correlated with the presence of soluble factors found in serum or other biological fluids of tumor-bearing hosts (Baroni et al. 1988). The work performed and reported from our laboratories has shown that H238 conditioned medium (CM) was able to decrease mouse splenocyte and human mononuclear cell (MC) proliferation in a dose-dependent manner (Prabhu Das et al. 1991). Such results indicated that the effects of TGF-β are not species-specific. Our laboratory has also shown that neutralization of concentrated H238 CM with monoclonal antibody to TGF-β resulted in complete abrogation of suppressive activity in spleen cell lymphoblastogensis (Prabhu Das et al. 1991). These results suggest that TGF-β may be the main inhibitor of immune responses found in the H238 tumor cell line. However, i.p. administration of polyclonal anti-TGF-β antibody did not significantly inhibit H238 tumor progression in vivo (Gridley et al. 1992). These results (Fig. 1 and 2) suggest

that other inhibitors of immune responses might be involved in H238 tumor progression in addition to TGF-β. This treatment with anti-TGF-β antibody, surprisingly, allowed tumors to develop as rapidly as and achieve greater volumes than the untreated H238-injected controls.

This current study reports that TGF-β significantly inhibits resting macrophage cytolytic activity against H238 tumor cells. After 18-24 hours of incubation with 1 ng/ml TGF-β, the cytotoxicity of macrophages was significantly reduced 10-15% compared with cells incubated with medium alone. When macrophages were incubated with 5-20 ng/ml TGF-β, the killing potential was decreased 25-50% compared with macrophages incubated with medium alone (Fig. 3 and 4B). These results suggest that TGF-β significantly inhibits the cytolytic activity of resting macrophages in a concentration-dependent manner. When the cytolytic activity of resting macrophages from 15 week-old mice are compared with that of resting macrophages from 11 week-old and 9 week-old mice, the results indicate that

macrophages from mice of different ages show variable killing activity and different sensitivity to TGF-β. The most interesting aspect to these studies was that TGF-β has little or no inhibitory effect on proteose peptone-activated macrophages (Fig. 4A). This mechanism is still unknown, but it might occur through interactions of other certain cytokines to prevent TGF-β from binding the specific receptors of TGF-β on macrophages when macrophages are activated by proteose peptone in vivo. When macrophages were treated with 5 ng/ml TGF-β and IFN-γ or IL-4 simultaneously, inhibition in the killing activity of resting macrophages was reversed 14-30 % compared with macrophages incubated with TGF-β alone (Fig. 6). These results also show that reversal by IFN-γ or IL-4 is a dose-dependent function.

TGF-β is known to be a powerful immunodulatory agent. It inhibits IL-2-dependent T cell proliferation (Kehrl et al. 1986); IL-1-dependent murine thymocyte proliferation; B cell proliferation and Ig secretion; and IFN-α, but not IL-2 enhancement of NK cell activity (Alain et al. 1986). Some studies have reported

that TGF-β also inhibits production of IFN-γ by peripheral blood mononuclear cells (Chentry et al. 1989), and the generation of nitric oxide (NO) by cytokine-activated macrophages (Nelson et al. 1991). These findings indicate that perhaps TGF-β exerts its effects through blockade of cytokine production or NO production. In the experiment of TNF-α production, the result (Table 1) showed that H238 tumor-bearing mice produce more TNF-α than normal control animals, mice treated with anti-TGF-β produced slightly more TNF-α than H238 tumor-bearing mice without any treatment. It is tempting to speculate that TGF-β produced by H238 tumor cells inhibited the secretion of TNF-α. However the data shown in Figures 1 and 2 indicate that anti-TGF-β antibody at the time-dose regimen used, has little or no inhibitory effect on H238 tumor progression in phase I and II in vivo. These results suggest that the suppressive effect of TGF-β on immune responses is complicated; certain other inhibitors from H238 tumor cells might also be involved in suppression of host immune responses, and inhibition of TNF-α production might be one of biological activities of

TGF-β.

The mechanisms by which TGF-β suppressed cytolytic activity of resting macrophages is not clearly known, but perhaps one mechanism may be to inhibit production of TNF-α or IFN-γ. Although TNF-α production is not unique to a particular cell type, the major source of this cytokine is the macrophage (Le et al. 1987). The biological activity of TNF-α has been demonstrated by its cytotoxic effects on certain sensitive target cells including the L929 fibroblast-like line and U937 cells in vitro. Studies have also shown that TNF-α can destroy tumors in vivo even in the absence of a direct lytic effect on neoplastic cells in vitro (Palladino et al. 1987). The data in the present (Table 1) shows that in vivo, the production of TNF-α may be suppressed by TGF-β secreted by H238 tumor cells. This suggests that one of the inhibitory effects of TGF-β on macrophage cytolytic activity may be through blocking TNF-α production by macrophages.

In addition to TNF-α the suppressive effects of TGF-β on killing activity of macrophages may be associated with IL-4 or IFN-γ production by T cells. The biological activities of IL-4 involve a wide range of cell types and functions that are only partially understood. IL-4 has been shown to promote T-cell growth (Sideras et al. 1988), IgG and IgE secretion by B cells (Snapper et al. 1988), and production of mast cell growth factor. It exerts its biological activity through a specific high affinity receptor expressed by cells of hematopoietic lineage including resting T cells, B cells, mast cells, myeloid progenitors, and macrophages (Park et al. 1987). Recent evidence suggests that IL-4 promotes the differentiation of macrophages into a cytotoxic phenotype (Crawford et al. 1987). IFN-γ not only induces class I and class II MHC antigens on many cells, but it stimulates some B cells, enhances IgG_{2a} production, and inhibits IgG_1 and IgE production. It also inhibits proliferation of Th2 cells and is a potent macrophage activator (Murray et al. 1985). The results shown in this study indicate that the inhibitory effect of TGF-β exerted on macrophages was reversed 14-30% in the

presence of IFN-γ or IL-4 and that TGF-β had no or little suppressive effect on peptone-activated macrophages. These data are consistent with the premise that IL-4 and IFN-γ both can activate macrophages into a cytotoxic phenotype in spite of the presence of TGF-β. They also suggest that another mechanism of suppressive effects of TGF-β on macrophage cytolytic activity may involve a blockade in IFN-γ or IL-4 production.

In addition to TGF-β IL-10, produced by the Th2 subset of $CD4^+$ T lymphocytes, $Ly\text{-}1^+$ B cells, and mast cells, is a newly discovered immunoregulatory cytokine of down-regulation (Sher et al. 1991). Some studies show that the major function of IL-10 appears to be to inhibit production of IFN-γ by T cells, production of IL-1, TNF-α, and IL-6 by LPS-activated macrophages (Fiorentino et al. 1991). Investigators have also reported that IL-10 inhibits parasite killing and NO production by IFN-γ-activated macrophages. These data indicate that IL-10 is a potential inhibitory cytokine. The data in this study, shown in Table 4 (Appendix) and Figure 5, confirm that IL-10 inhibits

macrophage cytotoxic activity. However, significant suppression was seen only at the highest dose of IL-10 used (50 ng/ml), and that the suppressive effect of IL-10 occurs in a dose-dependent manner. The inhibitory effect of IL-10 on macrophage function may be through blockade of TNF-α as well.

In this study, we have shown that IL-10 and TGF-β inhibit the cytolytic activity of resting, but not activated-macrophages against H238 tumor cells and that TGF-β appears to inhibit the production of TNF-α in H238 tumor-bearing mice. If IL-10 and TGF-β both inhibit the secretion of TNF-α, which is endogenous pyrogen, by macrophages, these two cytokines may play an important part in inflammatory responses by regulating macrophage function. TGF-β may be the most important inhibitor derived from H238 tumor cells as well as other tumor cells in order to prevent host immune responses.

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APPENDIX

Table 2. Suppressive effect of TGF-β on Murine Resting Macrophages

[a]Macrophage Treatment	Mean dpm ± S.E[b]. (X 1000)	% Killing[c]
Untreated (Control)	134.3 ± 8.3	76.0%
1.0 ng/ml TGF-β	112.8 ± 4.6*	62.6%
2.5 ng/ml TGF-β	106.2 ± 3.1*	58.5%
5.0 ng/ml TGF-β	93.5 ± 2.0*	50.6%
10.0 ng/ml TGF-β	89.8 ± 1.9*	48.3%
20.0 ng/ml TGF-β	100.3 ± 6.3*	54.8%

[a]Macrophages were obtained from 11 week-old mice.

[b]S.E. = standard error of the mean.

[c]Killing activity of macrophage was measured by % lysis of ^{3}H-thymidine labeled H238 cells.

*Significantly different from control macrophages ($p < 0.05$).

Table 3. Effect of TGF-β on Peptone-Activated Macrophages

Macrophage Treatment	[a]Active Macrophage		[b]Resting Macrophage	
	Mean dpm ± S.E[c]. (X 1000)	% Killing[d]	Mean dpm ± S.E. (X 1000)	% Killing
Untreated(Control)	238.2 ± 4.2	44.3%	187.2 ± 2.3	29.4%
1.0 ng/ml TGF-β	230.2 ± 4.6	42.0%	158.5 ± 6.4	21.0%
2.5 ng/ml TGF-β	233.1 ± 4.4	42.9%	161.2 ± 4.1	21.8%
5.0 ng/ml TGF-β	226.9 ± 6.9	41.0%	129.1 ± 4.1*	12.3%
10.0 ng/ml TGF-β	229.1 ± 3.5	41.7%	122.0 ± 4.2*	10.3%
20.0 ng/ml TGF-β	223.2 ± 6.6	40.0%	115.3 ± 3.1*	8.3%

[a]Active macrophages were obtained from 9 week-old mice that were injected 2 days earlier with 10 % proteose peptone.

[b]Resting macrophages were obtained from untreated 9 week-old mice.

[c]S.E. = standard error of the mean.

[d]Killing activity of macrophages was measured by % lysis of ^{3}H-TdR labeled H238 cells.

*Significantly different from control ($p < 0.05$).

Table 4. The Effects of IL-10 on Murine Macrophages

[a]Macrophage Treatment	Mean dpm ± S.E.[b]	% Killing
Untreated (Control)	167.3 ± 18.5	62.0%
0.5 ng/ml IL-10	172.7 ± 5.0	65.1%
5.0 ng/ml IL-10	167.7 ± 13.2	62.2%
10.0 ng/ml IL-10	159.9 ± 8.3	57.8%
25.0 ng/ml IL-10	151.2 ± 8.9	52.8%
50.0 ng/ml IL-10	121.8 ± 0.2*	35.9%

[a]Macrophages were obtained from untreated 12 week-old mice.

[b]S.E. = standard error of the mean.

[c]Killing activity of macrophages was measured by % lysis of ^{3}H-TdR labeled H238 cells.

*Significantly different from control value ($p < 0.05$).

Table 5. The Reversal of Inhibitory Effect of TGF-β on Macrophages by IFN-γ or IL-4

[a]Macrophage Treatment	Mean dpm ± S.E.[c] (X 1000)	% Killing[d]
Untreated	377.6 ± 18.9	100.0%
[b]TGF-β control	216.4 ± 26.8	50.6%
[b]TGF-β + 400 ng/ml IFN-γ	316.6 ± 32.4	82.3%
[b]TGF-β + 40 ng/ml IFN-γ	285.9 ± 5.5	72.6%
[b]TGF-β + 4 ng/ml IFN-γ	253.4 ± 29.8	62.3%
[b]TGF-β + 0.4 ng/ml IFN-γ	258.7 ± 26.2	64.0%
[b]TGF-β + 200 ng/ml IL-4	293.7 ± 19.3	75.0%
[b]TGF-β + 20 ng/ml IL-4	280.6 ± 28.1	70.9%
[b]TGF-β + 2 ng/ml IL-4	289.0 ± 0.7	73.5%
[b]TGF-β + 0.2 ng/ml IL-4	270.9 ± 5.7	67.8%

[a]Macrophages were obtained from untreated 15 week-old mice.

[b]TGF-β concentration was 5.0 ng/ml

[c]S.E. = standard error of the mean

[d]Killing activity of macrophage was measured by % lysis of ^{3}H-TdR labeled H238 tumor cells